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Thermodynamic Contributions of Various Metal-Nucleotide Complexes Binding to Yeast Phosphoglycerate Kinase

Randall Allen Holzberger
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Randall Allen Holzberger entitled "Thermodynamic Contributions of Various Metal-Nucleotide Complexes Binding to Yeast Phosphoglycerate Kinase." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Engin Serpersu, Major Professor

We have read this thesis and recommend its acceptance:

Elizabeth Howell, Chris Dealwis

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Thermodynamic Contributions of Various Metal-Nucleotide Complexes Binding to Yeast Phosphoglycerate Kinase

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Randall Allen Holzberger
May 2005

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Abstract

The purpose of this study was to determine the thermodynamic parameters of various enzyme-substrate complexes of Yeast Phosphoglycerate Kinase by using Isothermal Titration Calorimetry (ITC). The addition of metal-nucleotide substrate to a PGK solution elicited an exothermic response ranging from 0.5 kilocalories to 2.8 kilocalories. Titrations of MgATP and MgADP into PGK yielding binary complexes generated a greater amount of heat than titrations of MgADP into PGA-PGK and MgAMP-PCP into PGA-PGK yielding ternary complexes. The entropic contribution of the ternary complex formations was significantly greater than binary complex formations. Although entropic and enthalpic contributions varied, the Gibbs free energy remained relatively constant for the binary and ternary enzyme-substrate complexes. This data suggests that the entropic contribution becomes more dominant when PGA was added to form ternary complex of enzyme and ligand.

The titration of sugar substrate, 3-phosphoglyceric acid (PGA), into a PGK solution yielded an endothermic signal. The magnitude of the thermodynamic parameters remained elusive however. Numerous different regulatory molecules, which share many of the ionic characteristics of PGA, have been demonstrated to bind PGK in as many as six different regions within the catalytic core and along the periphery of the protein. As a result, titrations of PGA into PGK did not yield interpretable thermodynamic data. Enthalpy is a global entity encompassing the heat change of the entire system, and

consequently each titration likely included PGA binding at the desired active site in addition to a combination of other binding events at different areas of the enzyme.

Finally, the addition of sulfate to the various PGK complexes increased the dissociation constant of most of the substrates from the binary and ternary enzyme-substrate complexes and altered all thermodynamic properties of these complexes.

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List of Figures

PGK	Yeast Phosphoglycerate Kinase
1,3-BiPG	1,3-Bisphosphoglycerate
PGA	3-Phosphoglycerate
MgATP	Magnesium bound Adenosine Triphosphate
MgADP	Magnesium bound Adenosine Diphosphate
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
ITC	Isothermal Titration Calorimetry
DSC	Differential Scanning Calorimetry
Lys	Lysine
Arg	Arginine
His	Histidine
Asp	Aspartate
Glu	Glutamate
NADH	Nicotinamide Adenine Dinucleotide
MgAMP-PCP	Magnesium bound β, γ–Methyleneadenosine 5'-triphosphate

Chapter 1: Introduction

Phosphoglycerate Kinase (PGK) catalyzes the reversible transfer of a phosphate from 1,3-Bisphosphoglycerate (BisPGA) to MgADP yielding 3-Phosphoglycerate (PGA) and MgATP (see Figure 1). It is the first ATP producing step of Glycolysis and PGK is one of the oldest housekeeping enzymes in nature. PGK is a monomeric protein with a mass of approximately 46kDa, composed of two globular domains of approximately equal mass separated by a large crevice**.**

The PGK active site is located within the interdomain cleft and includes a separate binding site for each of the substrates located along the inner portion of the opposing lobes. The metal-nucleotide substrate has been suggested to bind within a shallow depression lining the interior surface of the C-terminal domain (Watson et al., 1982)**,** at a nucleotide binding site bearing typical nucleotide binding motifs (see Figure 2). The sugar substrate molecule (PGA) has been determined to associate adjacent to a series of residues, referred to as the "basic patch", located along the opposing face of the N-terminal domain (see Figure 3; Harlos et al., 1992).

Crystal structures from various PGK species remain slightly convoluted and the exact orientation of the substrates along with the coordinated side chains remained unclear for

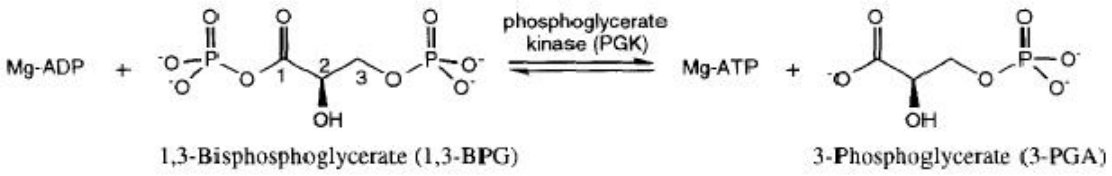

Figure 1 Reaction catalyzed by Phosphoglycerate Kinase

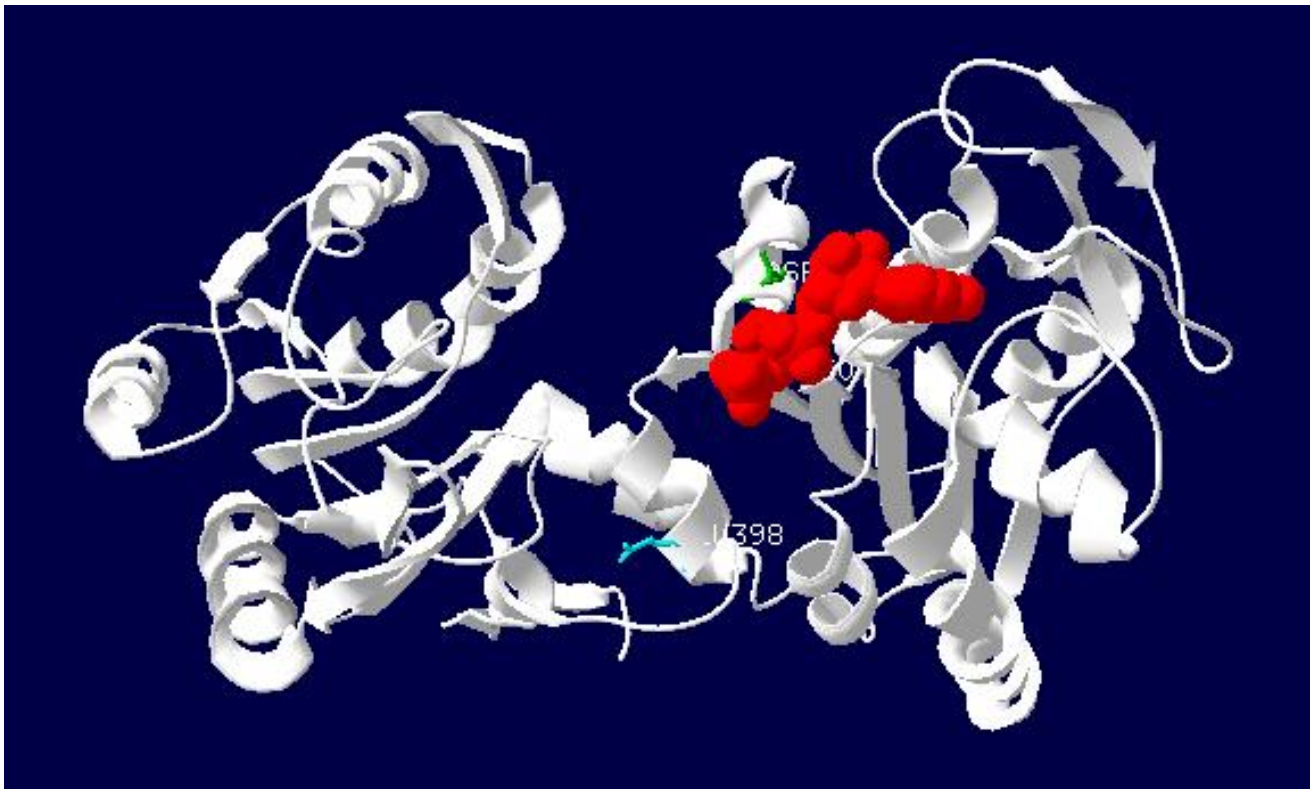

Figure 2 Crystal structure highlighting the binding orientation of the metal-nucleotide substrate to PGK along the C-terminal domain.

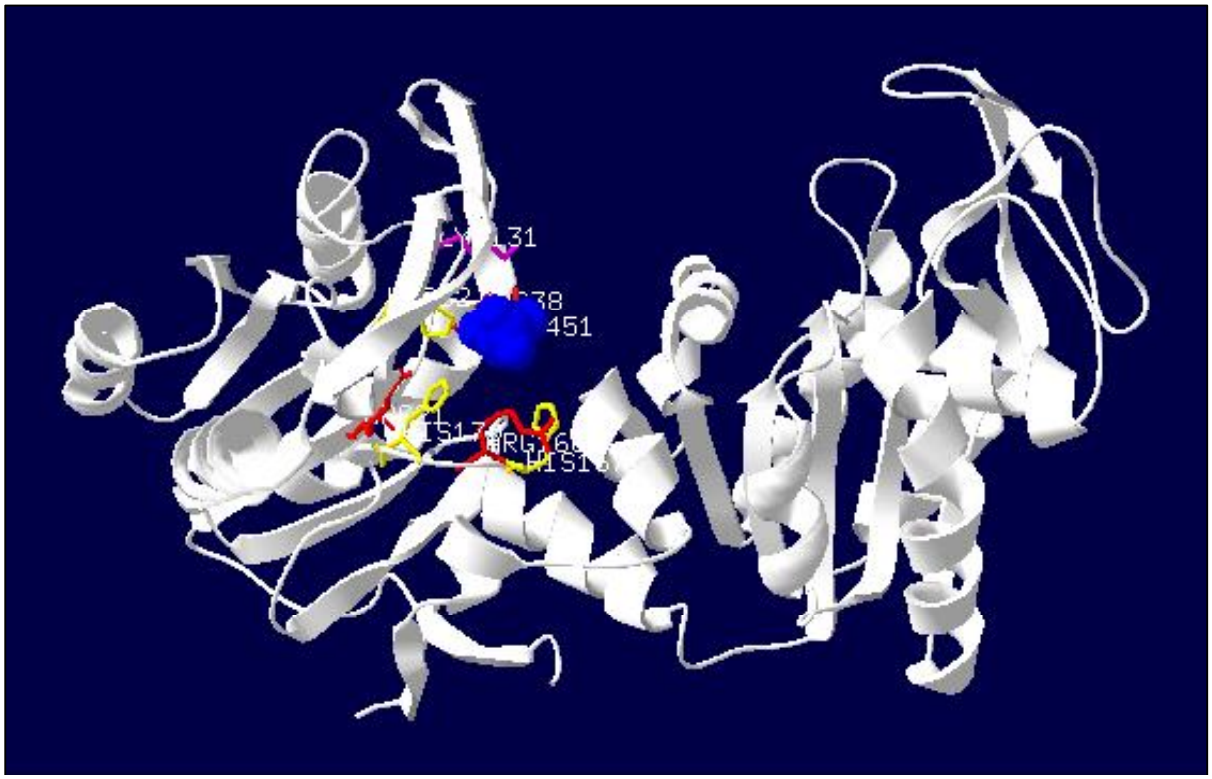

Figure 3 Crystal structure highlighting the binding orientation of the sugar substrate to PGK along the N-terminal domain

decades (Harlos et al., 1992; Banks et al., 1979; McPhillips et al., 1996; Bernstein et al., 1997).

The current structural understanding of PGK has largely become available only recently as the crystallization of ternary enzyme-metal ATP-PGA complexes was achieved. The ability to incorporate coordinated substrates simultaneously into the crystal model has provided unparalleled insight into the binding regions of enzyme as well as mechanistic clues regarding catalysis. X-ray crystallographic studies determined the structure of the PGK enzyme existing in a conformation orienting the substrate binding regions approximately 10Å apart (Banks et al., 1979; Watson et al., 1982). Mechanistic studies suggested a direct phosphoryl transfer between the substrates by possibly an inversion of configuration excluding a substrate intermediate (Webb and Trentham, 1980; Pappu et al., 1994). However, when bound, the substrates remained too distant for a simple transfer mechanism. Consequently, it was suggested that catalysis occurs in a "closed" state of the protein, which may form through a "hinge-bending" motion that brings the two domains of the protein together (Banks et al., 1979) very similar to that of the previously reported for hexokinase (Bennet and Steitz, 1978). A simplistic interpretation of the mechanism of catalysis from early crystal structures would suggest that the presence of both types of substrates is required for any domain movement resembling the hinge-movement. This is consistent with the conclusions of Pickover et al., (1979) and of Roustan et al., (1980) who utilized x-ray scattering and sedimentation equilibrium measurements, respectively, to determine that according to a large change in the

hydrodynamic properties of the active site upon the addition of both substrates, a major structural alteration occurs. Later, Henderson et al., (1994) determined that according to neutron scattering experiments, the radius of gyration of the protein decreased slightly upon the addition of each substrate as well as sulfate. The apparent compaction of the enzyme represented further evidence of major structural changes upon substrate binding. However, a crystal structure of the yeast enzyme solved by McPhillips et al., (1996) bound with both substrates remained in the "open conformation" with the substrates too distant for transfer. As a result, the key to the initiation of the hinge-bending motion remained elusive.

Most conclusions regarding the catalytic mechanism and instigation of the hinge-bending motion necessary for catalysis of the PGK reaction were as a result of kinetic studies, NMR experiments, x-ray and neutron scattering work, and crystal structures of the enzyme in the "open conformation." Most recently however, the crystal structure of *Trypanosoma brucei* PGK was solved at a resolution of 2.8Å (Bernstein et al., 1997). The *Trypanosoma* PGK ternary complex exhibited a dramatic closing of the large cleft between the two domains seemingly confirming all previous hypotheses and observations. The crystals displayed a domain rotation of 32° with respect to the native horse enzyme. The differences in the orientation of the domains between the unbound enzyme and the ternary complex were calculated as much as 27Å (Bernstein et al., 1997). These studies indicated that small conformational changes induced by the binding of the sugar substrate PGA, "prime" the PGA-bound enzyme to react differently to the binding of the metal-nucleotide (Bernstein et al., 1997). Upon binding, helix 14 was implicated

in a translational movement which, when combined with metal-nucleotide substrate association, created a hydrogen bonding pattern which resulted in the locking of the amino- and carboxy-terminal domains together in a new, more compact orientation. While the mechanistic and structural information confirmed previous insights, the conclusions must be tempered. *Trypanosoma* is an organism that relies solely on glycolysis to maintain homeostasis. Therefore PGK of this species catalyzes reactions at a higher rate versus a non-glycolytic species. Also, contrary to earlier crystal studies of the enzyme-PGA-Mg/MnAMP-PNP complex, the crystal structure of *T. brucei* PGK appeared to be in the catalytically active, closed conformation even though only associated to the metal-nucleotide substrate, MgADP. An amino acid sequence unique to *Trypanosoma* was inserted (residues 69-84) within the amino-terminal domain which could be responsible for inducing the closed conformation of the enzyme even in the absence of ternary complex formation.

Anion Binding to PGK

Many types of anions can associate and activate PGK to varying degrees. Considering both catalytic substrates (PGA and MgATP) and many regulatory substrates possess negative charges of varying magnitude, the binding possibilities remain extremely complex. However, a linear relationship exists between the negative logarithm of the dissociation constant and the net anionic charge of the regulatory substrate and anion binding becomes considerably tighter as the charge of the anion increases (Wrobel & Stinson 1978). Therefore affinity for the enzyme is directly related to the overall charge

of the substrate molecule. Early solution studies conducted by Scopes (1978) determined that there are at least two and possibly as many as six distinct regions of the PGK molecule which have the ability to bind to regulatory molecules. Ultimately, the similar ionic specificity of the catalytic and regulatory binding regions only serves to further convolute the binding characterization of the enzyme.

The Sugar Substrate Binding Pocket-"Basic Patch"

Site-directed mutagenesis at the putative sugar-substrate binding pocket was performed to determine the relationships between structure and function of the individual residues. Point mutations surrounding the "basic patch" region of the catalytic center of the enzyme yielded results different than expected (Sherman et al., 1990; Walker et al., 1992; Barber et al., 1993). The mutations R21A, R21Q, H62Q, H167S and R168Q produced functional enzymes with a Michaelis constant similar to that of wild type PGK. On the other hand, R38A and R38Q mutations resulted in loss of activity by about four orders of magnitude indicating that only R38 is critical for catalytic function. The H62Q mutant showed very similar kinetic behavior to the wild type enzyme with the addition of sulfate at various concentrations. Finally, the mutations H167S, R168Q, and R21A eliminated the activation witnessed at high concentrations of either MgATP or PGA. The results indicated that these PGK mutants displayed decreased anion activation while maintaining the K_m value similar to that of the native enzyme. These studies confirmed the location of an anion regulatory site near the periphery of the sugar substrate binding region.

Mutants R65Q, R65A, and R65S all displayed very similar kinetic properties (Sherman et al., 1991). The maximum velocities of the mutants excluding activating sulfate increased 100% from the wild type enzyme and the K_m values become smaller for both MgATP and PGA (Sherman et al., 1991). The decrease in excess substrate activation as well as sulfate activation implies that R65 plays a role in the anionic activation mechanism. It is clear from the comprehensive mutagenesis studies of the "basic patch" that only R38 plays a significant role in catalysis, while many of the adjacent residues are mainly involved in a regulatory mechanism.

While many of the residues of the basic patch have been implicated to possess a regulatory function via the mutagenesis work mentioned above, it has also been suggested that sugar substrate binding plays a critical role in triggering the hinge bending motion necessary for catalysis (Bernstein et al., 1997). To clarify the effect of binding of PGA at the active site, the relaxation rates between the paramagnetic substrate analog CrATP and inorganic phosphate were determined in the presence and absence of PGA. According to ^{31}P NMR spectroscopy, the distances between P_i and Cr^{3+} were calculated to be 6.9±0.5Å with bound PGA and 5.0±0.3Å in the absence of PGA (Serpersu et al., 2002). The change in the immediate active site environment due to PGA binding suggested that either the association of PGA to the basic patch altered the conformation of enzyme or a regulatory molecule, phosphate, may occupy the sugar substrate binding region prior to PGA addition and subsequently moved to an adjacent regulatory site upon sugar substrate binding. These results are consistent with Fairbrother et al., (1990b) who demonstrated a change in the solvent accessibility of the active site upon PGA binding.

Further NMR spectroscopic work isolated the interactions of sugar substrate as well as other anions to mainly R65 and R168, confirming previous studies (Fairbrother et al., 1990). The binding of PGA and BisPGA however, was stronger than expected on the basis of anion charge and causes conformational changes in the protein not seen with any of the other anions. These results are consistent with the binding studies by Wrobel and Stinson (1978) determining the relationship of the specificity of anion binding to the overall negative charge of the molecule. These results are also in agreement with earlier NMR studies determining a perturbation of the immediate environment of the metal-nucleotide substrate upon titration with PGA (Gregory and Serpersu, 1993). Without question, the addition of PGA to the active site instigates many of the interactions which are likely necessary for domain closure.

Metal-Nucleotide Binding

Unlike the ambiguity still witnessed concerning sugar substrate association, it has been long acknowledged that the metal-nucleotide substrate associates to a defined region of the Carboxy-terminal domain. The model of the yeast PGK molecule revealed the binding of the adenine group to a marked depression along the C-terminal lobe of the enzyme surface (Watson et al., 1982). The boundaries of the adenine binding hollow are defined by G211, L311, and V339. The results of a more recent crystal structure of pig muscle PGK were consistent with Watson et al., (1982) determining the adenine ring was bound in a hydrophobic slot on the surface of the carboxy-terminal domain of the enzyme (May et al., 1996). Initially, the metal-nucleotide substrate was also determined to

include a direct interaction with the carboxyl group of Asp372 (Watson et al., 1982). However, mutagenesis studies investigating the function of Asp372 raised questions regarding binding (Minard et al., 1990). Asparagine substitution at residue 372 decreased the maximum velocity of the reaction, but substantially less than one would expect from its known position within the structure of the enzyme. This result suggested that while Asp372 is significant for binding, it is not the sole residue responsible for metal-nucleotide substrate interaction. The specific role of Asp372 as the sole coordination site was also brought into question by proteolytic cleavage experiments (Pappu et al., 1997). After enzyme inactivation with the non-hydrolysable ligand RhATP and subsequent cleavage of the protein, NMR studies determined that E398 was also strongly coordinated to the metal-ion of the ligand. Therefore it was concluded that both D372 and E398 are critical residues for the binding of substrates and phosphate exchange of the PGK reaction.

Further evidence existed of multiple nucleotide binding regions within the catalytic core of the enzyme. An initial ATP binding site was determined which involves electrostatic interactions between the nucleotide and arginines of the "basic patch" region (Fairbrother et al., 1990). With the addition of increasing metal ion, a secondary site was elucidated characterized by predominantly hydrophobic interactions coordinating the adenosine molecule of the nucleotide and the protein (Fairbrother et al., 1990b; May et al., 1996). NMR studies confirmed that the secondary site determined by Fairbrother was indeed the catalytic site elucidated by the crystallographic structure solved by Watson et al., (1982).

It is likely the primary site represents a regulatory binding pocket while the secondary site is indicative of the catalytic core of the enzyme.

Role of Metal Ion for Nucleotide Binding

The role of the metal ion during catalysis has been studied at length. It has been demonstrated that metal-free nucleotide can associate with the enzyme at a site near the catalytic core of the enzyme (Scopes, 1978). Magnetic resonance studies involving measurements of the relaxation rates of water protons indicated that phosphoglycerate kinase belonged in the same class of kinases with maximal binding of metal ion to the enzyme in the presence of the nucleotide substrate. The metal-nucleotide substrate has an increased affinity of almost two orders of magnitude for the enzyme over the free nucleotide (Chapman et al., 1977). Corroborating evidence of enhanced nucleotide binding in the presence of metal ion was illustrated by Fairbrother et al., (1990b) who demonstrated by NMR that the affinity of the catalytic site is enhanced relative to the primary nucleotide site with increasing Mg^{2+} concentration. Clearly, the metal ion performs a critical role in the coordination and proper orientation of the nucleotide for binding and subsequent phosphate transfer. In addition to aiding nucleotide binding, Pappu et al., (1994) suggested that the metal ion may also assist in the alignment of both substrates for phosphoryl transfer and may provide a mechanism to initiate the hinge-bending motion for catalysis. Studies utilizing proteolytic cleavage of the RhATP-inactivated enzyme with pepsin determined a novel coordinating residue of the metal ion in complex with the enzyme and nucleotide substrate, E398 (Pappu et al., 1997). It is

possible that the metal-nucleotide substrate may be altering its coordination site to a different residue or residues as a result of ternary complex formation and the subsequent "hinge-bending" motion required for catalysis. Therefore the coordinating metal ion likely plays several important roles in catalysis including binding and altering intramolecular interactions.

It was hypothesized that the hinge bending motion was initiated by coordination of the metal ion to both the bound substrates simultaneously along their separate domains (Pappu et al., 1994). The coordination would then cause subsequent interdomain interactions to arise and lead to the closed active site. Crystal work on the non-productive ternary complex PGK-PGA-MnADP determined that the metal ion remained greater than 11Å apart, again too distant for direct phosphate transfer (Ray and Rao, 1988). Therefore the simple coordination to both substrates was not enough to initiate catalysis. It was concluded that perhaps the absence of the gamma phosphate of the nucleotide does not permit coordination and prevents domain closure.

A more recent crystallographic structure was solved by McPhillips et al., (1996), which included the substrates Mg-AMP-PNP and PGA associated to the Active Site concurrently. The substrate MgAMP-PNP included coordination of the metal ion to the α, β, and γ phosphates of the nucleotide in addition to Asp372. The results indicated the enzyme had failed to undergo the hinge-bending motion for catalysis as the substrates were still too distant for phosphoryl transfer and the enzyme remained in the "open conformation." As a result, it was suggested by McPhillips et al., (1996) that closure of

the domains was accompanied by a "twisting" motion instead of a simple compression. Solution studies were performed to further investigate the causes of the hinge bending mechanism and how metal ion coordination to the nucleotide affects initiation of the hinge-bending motion. It was determined that bidentate $Rh(H_2O)_4ATP$ over tridentate $Rh(H_2O)_3ATP$ was preferred by the enzyme for domain closure (Pappu et al., 1994). These results were consistent with the above observation since the coordination of the α–phosphate to the metal ion of the tridentate substrate results in a more compact structure versus the bidentate form. Therefore, the bound sugar substrate may only be able to reach and interact with the less compact bidentate substrate, which lacks coordination to the α–phosphate, initiating the hinge bending motion for domain closure. The coordination of the metal ion to only two of the associated phosphates of the nucleotide and the sugar substrate concurrently likely introduces the "specific interactions" suggested by May et al., (1996) necessary for initiation of the hinge-bending motion. Therefore, the coordination of the metal-ion plays a key role in the instigation of the "hinge-bending" motion for catalysis.

The Addition of Activating Sulfate to PGK

Effects of anions binding to phosphoglycerate kinase have been studied at great length**.** Most importantly, the inclusion of even small concentrations of sulfate to PGK complex formation can activate the reaction by as much as 400% (Scopes, 1978). Other anions such as phosphate and excess substrate have a similar activation effect. The exact mechanism how multivalent anions activate this enzyme is not known.

The kinetic interactions of the PGK reaction involving sulfate have been examined extensively. It was determined that at millimolar concentrations, sulfate acts to increase both the K_m and the V_{max} for the metal nucleotide as well as the sugar substrate (Khamis and Larsson-Raznikiewicz, 1981). Double-reciprocal kinetic graphs proved to be non-linear for each variable substrate (Scopes, 1978; Larsson-Raznikiewicz, 1967), sloping downward beyond catalytic site saturation indicating that substrate concentrations exceeding the threshold required for catalysis consistently activated the PGK reaction. The presence of sulfate linearized the Lineweaver-Burke plots indicating saturation of all excess binding pockets outside the catalytic core of the enzyme. Full activation of the enzyme was achieved through sulfate addition eliminating any regulatory role from excess substrate (Larsson-Raznikiewicz, 1967; Scopes, 1978).

Kinetic work also identified sulfate as an activator as well as an inhibitor of the PGK reaction (Larsson-Raznikiewicz and Jansson, 1973; Scopes, 1978). It was determined there are at least two distinct binding sites for Sulfate (Scopes, 1978), one at the catalytic center of the enzyme as well as a second anion binding region remote from the Active site (Khamis & Raznikiewicz 1981). One hypothesis is that because Sulfate performs inhibiting as well as activating tendencies, these regulatory sites mediate opposing functions. It is unclear which regulatory site is responsible for activation and/or inhibition. In order to elucidate the functional role of each site, it is necessary to examine activation and inhibition separately. Based on kinetic studies performed by Khamis and Raznikiewicz (1981), at substrate levels <0.5mM, moderate (2-10mM) sulfate ion reduced PGK activity. On the other hand however, once substrate concentrations were

increased from 0.5mM beyond 1mM, the reaction was activated by sulfate ion (Khamis & Raznikiewicz 1981) suggesting that the substrate was able to expel sulfate from the catalytic core of the enzyme. Furthermore, kinetic data by Scopes (1978) determined that sulfate inhibited competitively with both of the substrates individually. Thus, the presence of sulfate should affect binding of both substrates. Still little is known of the energetic consequences of substrate coordination in the presence of activating sulfate.

Investigation of the multiple anionic regulatory binding regions provided surprising insights. Sulfate possessed a greater affinity for the anionic regulatory binding region versus the catalytic site (Khamis and Raznikiewicz, 1981). Thus, at low substrate concentrations, sulfate was able to successfully compete with the catalytic substrates at the lower affinity regulatory site located within the catalytic center. With increasing substrate concentration however, substrate began to saturate the Active site and displace activating sulfate from the binding pocket. Therefore, at moderate sulfate concentration, activation of the PGK reaction is believed to be a result of both, decreased inhibition at the active site as well as the association of anion to a regulatory binding pocket remote from the interdomain cleft (Khamis and Raznikiewicz, 1981). This hypothesis provides a simplistic mechanism of activation of the enzyme, but is limited by the expansive poly-ionic nature of the protein.

Structural work by Fairbrother et al., (1990) confirmed a binding pocket for anionic molecules including sulfate, distant to the Active site through NMR studies utilizing paramagnetic probes. In addition, a secondary low-affinity site was defined which most

likely overlaps the catalytic core. Anion association to the primary site was demonstrated to be responsible for activation whereas association to the secondary pocket obstructs substrate binding, thus resulting in inhibition at higher anion concentrations (Fairbrother et al., 1990; Sherman et al., 1990). It was also demonstrated by Serpersu et al., (2002) that PGA and P_i can bind to the enzyme concurrently at separate binding pockets in close proximity to each other. The lengthening of the relaxation times of P_i upon PGA titration clearly indicated that the sugar substrate was displacing phosphate from the PGA binding site. However, the displacement does not extend too far as the phosphate still remains near the active site. These results are consistent with the preliminary structural and solution studies detailing partially overlapping binding sites for the sugar substrate and the regulatory substrate.

A further consequence of the introduction of sulfate into the PGK reaction is the decreased radius of gyration of the enzyme determined by neutron scattering studies (Henderson et al., 1994). Structural differences upon sulfate binding demonstrated a variation in the solvent environment of the interdomain cleft resulting in a proposed slight compression of the enzyme (Roustan et al., 1979). It is not fully understood how this more compacted conformation affects the binding or thermodynamics of the PGK reaction

Isothermal Titration Calorimetry

Isothermal Titration Calorimetry (ITC) provides a direct route to the complete thermodynamic characterization of non-covalent, equilibrium interactions. Direct measurement of the heat of interaction as one component is titrated into another enables the determination of the change in the observed calorimetric enthalpy, ΔH_{obs}. The strength of ITC lies in its unique ability to measure binding reactions by the detection of heat change during the molecular interaction. ITC is a sensitive, direct method of detection lacking the necessity of molecular immobilization and the associated difficulties. Recent advances in ITC instrumentation have facilitated the experimental designs that utilize the direct measurement of large binding affinities, the coupling of binding to protonation/deprotonation processes and the analysis of binding thermodynamics in terms of structural parameters (Leavitt and Freire, 2001). Because isothermal titration calorimetry has the capability to measure different energetic contributions to the binding affinity, it provides a unique bridge between computational and experimental analysis. Since heat changes occur during many chemical processes, ITC has a variety of applications ranging from biochemical studies to more complex processes involving enthalpy changes such as enzyme kinetics.

The thermodynamic contributions attributed to the binary and ternary complex formation of PGK have not been investigated thoroughly. As previously detailed, many types of ionic molecules can bind PGK separately or in complex (Wrobel and Stinson, 1978). The enthalpy change is a property of the entire thermodynamic system in solution. It

represents the total heat released or absorbed into the calorimetric cell upon each individual ligand titration. As a result of the nature of the enzyme to coordinate poly-ionic molecules encompassing both catalytic and regulatory substrates, the previous thermodynamic characterization of PGK almost certainly included some combination of binding events of the different substrates.

When simplifying the PGK reaction to a single ligand-substrate relationship, what are the individual direct thermodynamic contributions? How do the entropy and enthalpy components respond to different complex associations with PGK? What differences appear when comparing the formation of binary complexes (metal-nucleotide titration with PGK only) and ternary complexes (metal-nucleotide titration in the presence of PGK and PGA)? Equally as important, what is the effect of the activating anion sulfate on the binary and ternary complex formations and what type of insight does this shed on the overall reaction? Therefore, this study was undertaken to understand the thermodynamic properties of the enzyme-ligand complexes of yeast phosphoglycerate kinase complex.

Chapter 2: Materials and Methods

Isolation of PGK

Yeast phosphoglycerate kinase was isolated from yeast strain 20B-12 containing multicopy plasmid pCGY219 for overproduction, kindly provided by Dr. Hitzeman (formerly Genentech Inc.). The yeast strain was deficient in the Peptidase Four and Five genes in order to decrease protein degradation. In addition, the vector was engineered with Tryptophan synthesizing capability for selection. Yeast cells were cultured in complete media (Yeast Nitrogen Base with Cas Amino Acids which lacks Tryptophan) for maximum protein expression. Cells were plated on the previously mentioned media and scaled up utilizing a maximum dilution of 200:1 for intermediate cultures. Large-scale (10L) fermentation cultures were employed to maximize cell growth and subsequent protein production. Fermentation was performed at 30ºC, applying approximately 10psi of incoming filtered aeration. Cells were harvested by centrifugation, washed in sterile H_2O and stored at -80ºC.

The cells were lysed with a previously reported ammonia auto-lysis method (Kulbe and Bojanovski, 1982). The solution consisted of 1 mM PMSF, 3 mM EDTA, and concentrated ammonia diluted equally with the mass of the cell pellet. The resulting mixture was stirred for approximately eight hours and allowed to stand overnight at room temperature. The crude lysate was centrifuged at 13,000g for fifteen minutes followed by another centrifugation at 15,000g for one hour, both at 4ºC in order to remove large and small cellular bodies respectively. DNA was precipitated from the solution through the

addition of 0.1% protamine sulfate with stirring and subsequent separation by centrifugation at 13,000g for one hour at 4ºC**.** Further nucleotide degradation was achieved by DNAse/RNAse treatment. The protein was precipitated by Ammonium sulfate fractionation at 30% keeping the supernatant. The resulting protein solution was subjected to gel filtration chromatography using Sephacryl HR200S column (diameter: 2.5cm, height: approximately 90cm) equilibrated in 5 mM Tris-HCl, pH 7.5 at 4ºC collecting 5mL fractions. The enzyme was >95% pure as determined by SDS-polyacrylamide gel electrophoresis (Figure 4) using densiometry software from Lab Works (Upland, CA). Enzyme purity was confirmed by UV spectroscopic analysis. All protein fractions that possessed A_{280}/A_{260} absorbance ratio of >1.5 and show greater than 200 units/mg activity were retained. The resulting protein solution was concentrated by lyophilization and quantified by spectroscopic analysis at 280 nm utilizing an extinction coefficient of ε =0.5 equaling 1mg/mL protein concentration.

Activity Assay

PGK activity was spectrophotometrically determined by measuring the oxidation of NADH to NAD^+ in a coupled reaction with the enzyme glyceraldehyde phosphate dehydrogenase (see figure 5). The decrease in absorbance at 340nm indicated activity over time. In this assay, oxidation of one mol of NADH corresponds to hydrolysis of one mol of ATP.

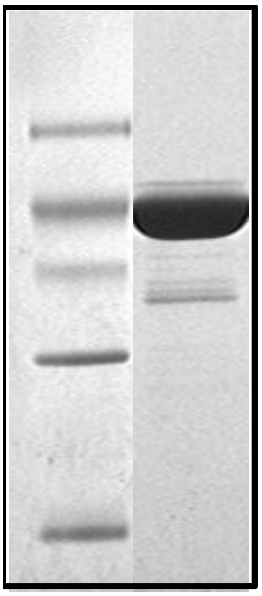

Fig. 4 12% SDS-PAGE displaying >95% purity of PGK using densiometry software from Lab Works, Upland, CA

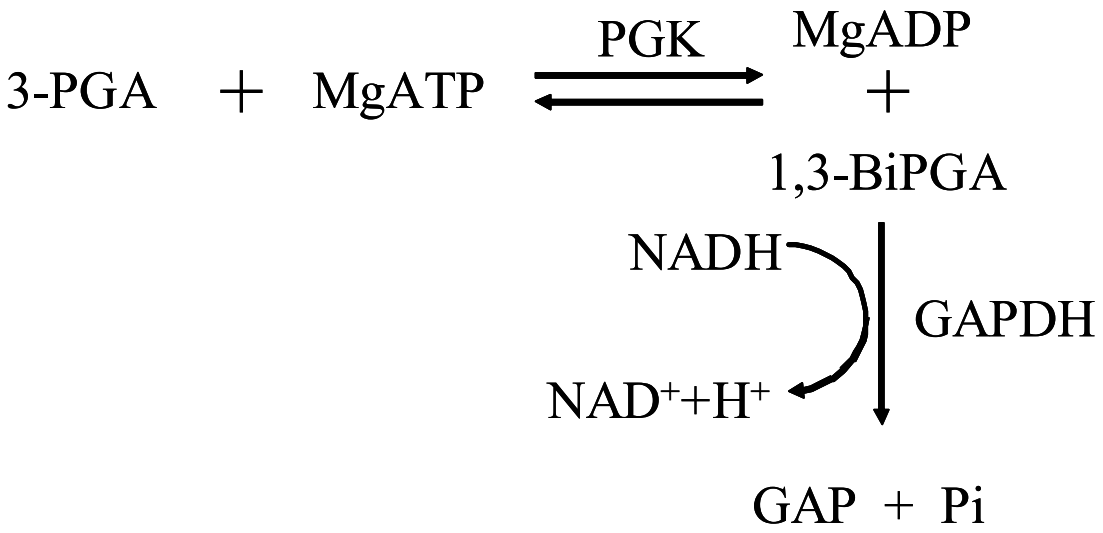

Figure 5 Diagram of PGK activity assay coupled to the oxidation of NADH by GAPDH. The stoichiometry of both reactions is 1:1 and therefore activity is measured by the decrease in NADH concentration.

Sugar Substrate Preparation

Phosphoglyceric Acid (PGA) was obtained commercially from Sigma-Aldrich as a Barium Salt. The removal of Barium was performed through the addition of concentrated hydrochloric acid to solubilize the insoluble Barium-PGA complex, followed by the addition of stoichiometric amount of concentrated sulfuric acid to precipitate the Barium in sulfate form, and then the solution was neutralized with sodium hydroxide. The solution was then applied to a Chelex 100 column to eliminate any remaining trace metal ions and stored at 4ºC.

Reverse Reaction

PGK is responsible for the orientation of the two substrates, MgADP and BisPGA, in a manner such that the C-1 phosphoryl group can be transferred from the high-energy sugar molecule creating the significantly more stable products PGA and MgATP. Considering the significantly increased stability of PGA over BisPGA, the reverse reaction was utilized in order to simplify manipulation.

GAPDH Preparation

Glyceraldehyde Phosphate Dehydrogenase (GAPDH) was obtained as a salt solution from Roche Chemicals. The enzyme was dialyzed against 5 mM Tris-HCl, pH 7.5 at 4ºC exhaustively to remove the remaining salt components and stored at -20ºC.

Isothermal Titration Calorimetry

Isothermal Titration Calorimetry was utilized to determine the thermodynamic contributions of each substrate in the different complexes of the yeast phosphoglycerate kinase reaction. Upon ligand addition to a substrate solution, the total heat Q of the system can be calculated relative to the concentration of both ligand and substrate within the cell.

Specifically:

$Q= n\Delta M_t \Delta H(V_0)$

Where:

n = number of sites

V_0= current cell volume

M_t= bulk concentration of macromolecule (substrate)

The binding constant for the system can be also calculated:

$K= \theta /((1-\theta)[X])$

Where:

X = concentration of the ligand

θ = fraction of sites occupied by ligand X

Combination and rearrangement of the equations allow for the calculation of the heat generated for the i^{th} injection for any given values of n, K, and ΔH. However, it is not the total heat, which is valuable for experimental data, but the change in heat. Since the volume of the cell is changing with each injection, the concentration of ligand and macromolecule is also changing. Therefore a correction must be made in the total heat equation per i^{th} injection.

$$\Delta Q_{(i)} = Q_{(i)} + (dV_i/V_o)[(Q_{(i)} + Q_{(i-1)})/2] - Q_{(i-1)}$$

Where:

V = active cell volume

The fitting software will then perform an initial estimate for the values of n, K, and ΔH. The $\Delta Q_{(i)}$ is then calculated for each injection and compared to the measured heat for each experimental injection. The parameters are varied for each iteration until no further significant improvement occurs.

The purpose of the fitting procedure is to determine the thermodynamic parameters (n, K, and ΔH), which best fit the data isotherm. The standard method of defining the "best fit" is to choose the parameters so that the sum of the squares of the difference between each point on the theoretical curve and the experimental curve reaches the minimum value.

The microcalorimetry data were collected at 28ºC (301K) using a VP-ITC system (see figure 6). Data was analyzed with the Origin 50 Software. A typical experiment consisted of the enzyme solution (classified as the substrate for the instrument), which remains in the calorimetry cell and a solution containing the ligand (classified as the ligand by the instrument), which is added to the reaction cell. A characteristic sequence involves 50 injections of 5µL each, lasting for 10 seconds totaling 250µL. With a space of 120 seconds between each injection, total experiment time lasts just under two hours. Initial volume of reaction cell was approximately 1.4mL prior to the first injection.

Enzyme concentrations were varied from 0.1 mM to 0.5mM and pH adjusted to 8.0. Variation of the macromolecule was correlated to the variation of the ligand in order to maintain the proper molar ratio of the experiment. For each experiment, the substrate, ligand, and buffer titration solutions were all prepared simultaneously. In addition to the reactants, all experimental solutions contained 5 mM Sodium Sulfate or 15 mM Sodium Chloride buffered with 50mM Tris pH 8.0. The pH of each solution and the activity of the enzyme was measured before and after each experiment to rule out pH change and enzyme inactivation during the experiments.

In order to analyze the raw data, the integration of each injection is calculated and adjusted according to the heat of dilution from a similar ligand titration into identical buffer components, excluding enzyme. The result was a net isotherm, which can then be fitted to an assortment of theoretical binding models. In all cases, a single site model yielded a better fit to data.

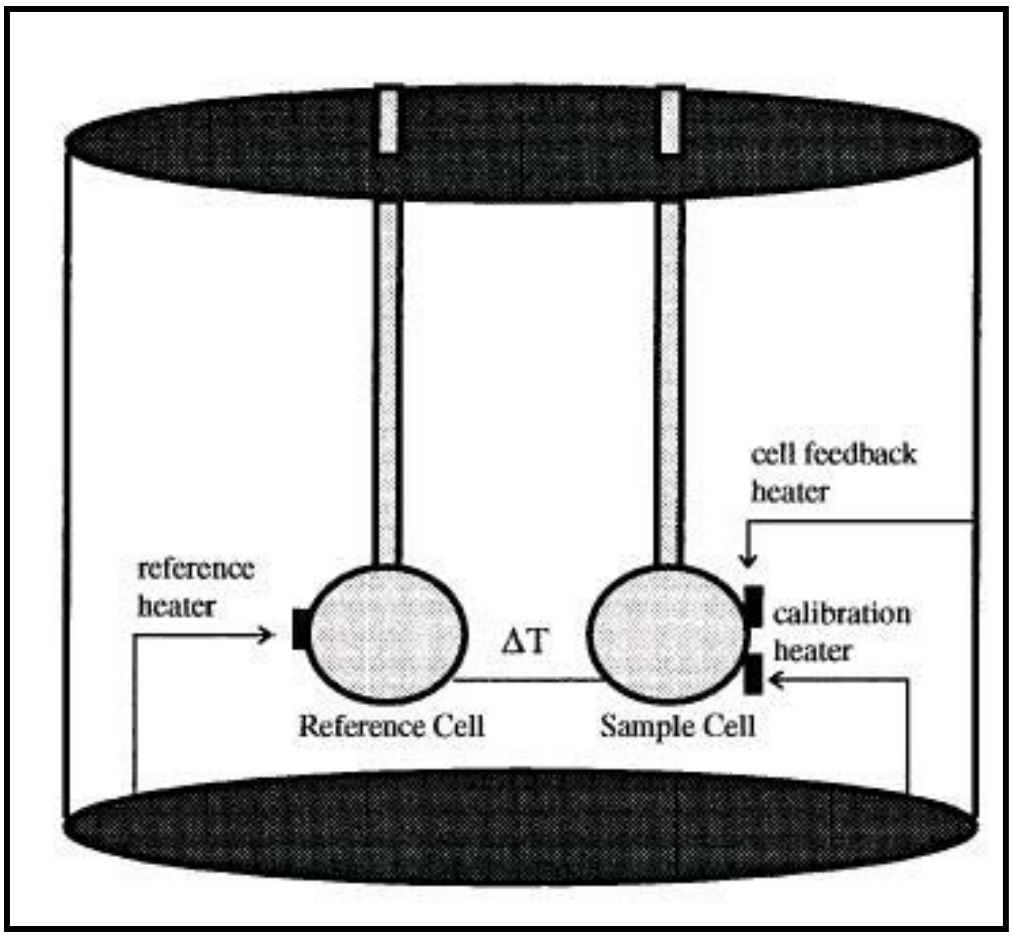

Figure 6 A schematic representation of an Isothermal Titration Calorimetry System

Chapter 3: Results

The interaction of biological molecules highlights the complexity and diversity of substrate-ligand interactions. It is of great interest to determine the nature of the forces that stabilize the interaction. The thermodynamics of binding are defined by the stoichiometry of the interaction, dissociation constant, enthalpy, entropy, and free energy of association. In this study, ITC was utilized to describe the interactions of the enzyme PGK and its ligands.

In general, the binding of PGK to its various metal-nucleotide substrates creating both binary and ternary complexes was consistently exothermic and entropically favorable. Formation of the PGK complexes was determined to be driven by both the enthalpy and entropy.

When considering the PGK reaction, the various types of regulatory substrates must be incorporated into the experimental strategy. Minimization of the lingering regulatory effect of excess substrate is vital to the discovery of the individual thermodynamic contributions of each titrant. Previous kinetic studies reported that multivalent anions can alter the activity of PGK even at small substrate concentrations (Schierbeck and Larsson-Raznikiewicz, 1979). Additionally, catalysis in the presence of excess substrate has been proven to produce non-linear double reciprocal kinetic plots (Scopes, 1978) confirming

activation of the enzyme. As a result, the ligand solution was composed of overwhelming metal ion to ensure there is very little if any free nucleotide present in solution to eliminate effects of anion binding to other site(s). Thus all experiments were performed under conditions where more than 95% of ATP was in the metal-nucleotide complex. Metal-ion titrations into PGK alone were performed and the thermodynamic contributions determined represented previous buffer titrations (data not shown) and were negligible.

Further steps were undertaken in order to minimize the environmental effects of the solution on the enzyme. It was determined that there are at least dual and perhaps as many as six regulatory binding regions adjacent and remote to the catalytic core of the interdomain cleft (Scopes, 1978). Inclusion of the activating anion sulfate performs an alternate function beyond the recognized regulatory role. The addition of sulfate serves to linearize double-reciprocal kinetic plots even in the presence of excess substrate (Scopes, 1978; Larsson-Raznikiewicz, 1967). While the mechanistic role of sulfate remains ambiguous, it is believed that at the appropriate concentration, activation is achieved through complete saturation of all regulatory sites within the interdomain cleft as well as along the external surface of the protein. The result is the full activation of the enzyme eliminating regulatory function from any excess substrate remaining in the solution environment creating a linear Lineweaver-Burke kinetic plot. Ultimately, the enthalpy detected from the titration involving the fully activated PGK enzyme bound to sulfate, should be limited to the singular interaction of the ligand and the substrate.

Optimizing the experimental conditions in order to produce the most efficient signal to noise ratio, proved to be very challenging. Previous solution studies have reported that even a small increase in ionic strength can lead to a difference in binding affinity by as much as an order of magnitude (Scopes, 1978). It was proposed that the added ionic strength of the solution disrupted the interactions of the charged side-chains with the highly polar substrates. The result was a "shielding effect" which negated much of the affinity of the individual substrates for their respected binding pockets (Scopes, 1978). It was also determined that ionic strength I > 1.0M decreased the individual titration response signal approximately four-fold (data not shown). However, the presence of salt within the experimental solutions remained in moderate concentrations in order to further minimize any non-specific ligand interactions. Therefore, for experiments excluding activating sulfate, sodium chloride was supplemented for sulfate ion in order to maintain consistent ionic strength I = 0.065M of the system. As a result, the variable of ionic strength was eliminated.

Binary Complex Formation in the Absence of Sulfate

The investigation of the thermodynamic interactions initiated by metal-nucleotide binding involved the titration of various ligand solutions (MgATP, MgADP, and MgAMP-PCP) into different PGK solutions containing or excluding sulfate ion. In this instance, the metal-nucleotide ligand was titrated into the macromolecule (PGK) solution at a 50:1 ratio to approach saturation indicated by negligent change in heat between injections. Structural work by Henderson et al., (1994) determined a slight domain

closure of the opposing lobes upon association to MgATP and MgADP individually. As a result, it is likely that environment of the active site is altered with the introduction of the ionic interactions associated with metal-nucleotide binding.

The initial complex investigated was the enzyme association with MgATP excluding sulfate. The first injection of metal-nucleotide solution into the enzyme solution yielded an exothermic response. The magnitude of ensuing injections decreased in intensity until apparent saturation was reached (see figure 7). Qualitatively, the isotherm represented a classic single binding site mechanism. After buffer correction, the data was fitted to a single site model utilizing the Origin 50 (Microcal) software and the thermodynamic parameters were determined.

The titration was exothermic, releasing a moderate amount of heat, ΔH = -2.3 kcal/mol as a result of complex formation. The ligand bound the enzyme with considerable affinity, K_D= 0.29 mM. The free energy of the substrate binding was determined to be highly favored, ΔG= -5.0 kcal/mol. Finally, the dispersion of energy of the reaction was calculated to be a favorable entropic value, ΔS= 9.0 $calK^{-1}mol^{-1}$.

Further exploration of PGK complexes led to the association of enzyme with MgADP excluding sulfate. The thermodynamic contributions for the diphosphate complex devoid of sulfate varied slightly with the observed values of the previous metal-nucleotide studies involving the triphospate titration. The association of MgADP, devoid of sulfate,

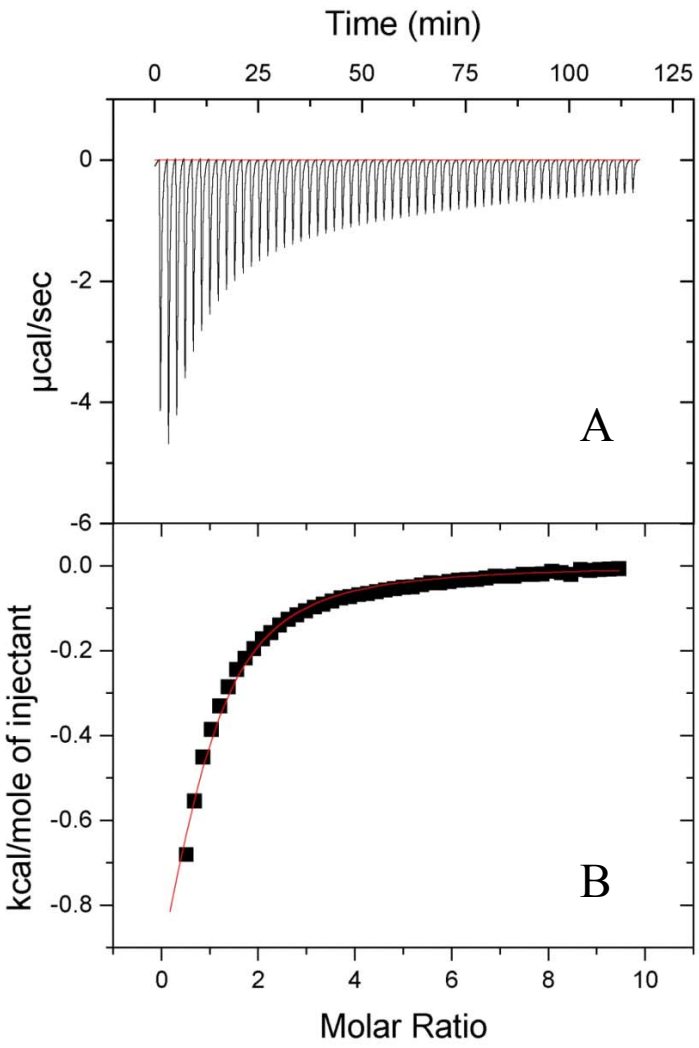

Figure 7 **Binding of MgATP to PGK without Sulfate.** *A*, A calorimetric profile of 5µL aliquots of 25 mM MgATP into 0.5 mM Phosphoglycerate Kinase in 50mM Tris, pH 8.0 in the presence of 15 mM NaCl. **Isothermic representation of MgATP binding to PGK.** *B*, A least squares fit of the data to the heat absorbed per mol of titrant versus the ratio of the total concentration of MgATP to the total concentration of Phosphoglycerate Kinase.

generated slightly less heat than the MgATP titration, ΔH= -1.7 kcal/mol. The binding of the diphosphate, metal-nucleotide substrate, MgADP, displayed a decreased binding affinity, K_D=0.39 mM. The free energy change of the reaction remained nearly constant as the reaction was determined to be again spontaneous, ΔG= -4.8 kcal/mol, while the entropic contribution increased faintly versus triphosphate binding, ΔS= 10.1 $calK^{-1}mol^{-1}$.

Binary Complex Formation in the presence of Sulfate

The presence of sulfate was determined to saturate many of the non-specific, anionic regulatory sites, to which excess substrates possess affinity (Khamis & Larsson-Raznikiewicz, 1981). It was earlier determined that sulfate can strongly influence the shape of kinetic plots even at modest concentrations (Scopes, 1978). The typical non-linearity of kinetic curves is converted to linearity with the introduction of activating sulfate indicating saturation of excess binding pockets surrounding the enzyme eliminating enzyme coordination with excess substrate (Scopes, 1978; Khamis & Larsson-Raznikiewicz, 1981). Therefore, the presence of moderate sulfate should create a simplified binding characterization of the various PGK complexes.

The addition of sulfate during catalysis has multiple effects on the PGK reaction ranging from activation to structural alterations (Scopes, 1978; Henderson et al., 1994). The structural and kinetic changes due to the addition of sulfate are well-characterized; however, the full energetic effects of sulfate activation have not been investigated. The

titration with MgATP into PGK was also demonstrated to be an exothermic reaction (see figure 8), which released 1.6 kcal/mol of heat. The metal-nucleotide substrate titration exhibited a decreased affinity versus the metal-nucleotide titration excluding sulfate ion, K_D = 0.38mM. A favorable entropic contribution was determined $\Delta S = 8.1\ calK^{-1}mol^{-1}$, while the overall binding reaction was calculated to be exergonic with a favorable change in free energy ΔG =-4.1 kcal/mol.

The titration of the diphosphate metal-nucleotide, MgADP, into PGK with sulfate released slightly more heat than the triphosphate titration. For each binding event, the enthalpic contribution was again exothermic, liberating a greater amount of heat than the triphposphate titration, ΔH= -2.7 kcal/mol. The ligand affinity for the enzyme was calculated to be decreased versus the previous MgADP titration devoid of sulfate, K_D = 0.62mM. The association of the ligand prevailed as a result of the reaction remaining favored with, ΔG= -4.4 kcal/mol. The entropy of binding decreased from the previous titration, $\Delta S = 5.6\ calK^{-1}mol^{-1}$.

Non-productive Ternary Complex Formation

Inspection of the ternary complexes devoid of sulfate exhibited similar tendencies as the previously mentioned anion activated binary complexes. While structural changes occur upon single substrate coordination to PGK, the most remarkable alterations of the bilobal conformation are evident with ternary complex formation (Henderson et al., 1994). The decreased radius of gyration determined upon complete active site formation was

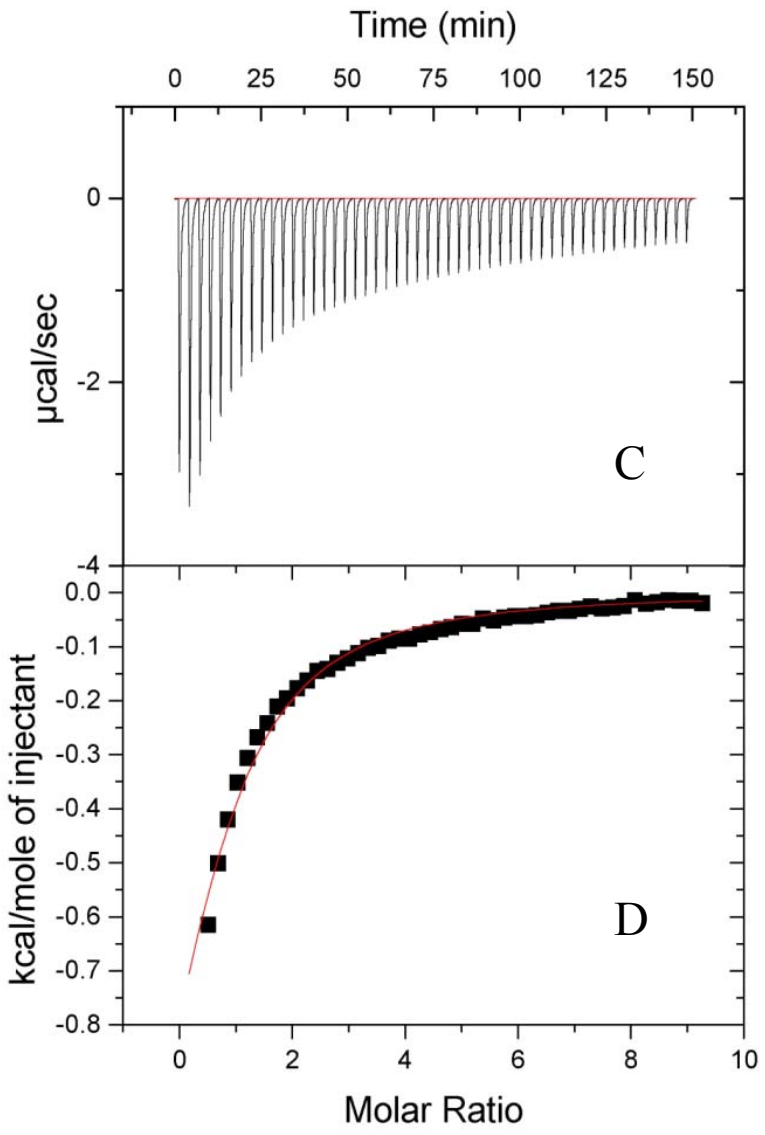

Figure 8 **Binding of MgATP to PGK with Sulfate.** *C*, A calorimetric profile of 5µL aliquots of 25 mM MgATP into 0.5 mM Phosphoglycerate Kinase in 50mM Tris, pH 8.0 in the presence of 5 mM Sulfate. **Isothermic representation of MgATP binding to PGK.** *D*, A least squares fit of the data to the heat absorbed per mol of titrant versus the ratio of the total concentration of MgATP to the total concentration of Phosphoglycerate Kinase.

consistent with the "hinge-bending" motion for catalysis suggested by Banks et al., (1979). The thermodynamics of these conformational changes upon ternary complex formation have not been examined thoroughly. According to structural work on the yeast enzyme, domain closure occurs upon ternary complex formation featuring metal coordination to the β and γ phosphates of the nucleotide only (Pappu et al., 1994). Therefore, while significant active site changes will be initiated with the addition of PGA to create the PGA-MgADP-PGK ternary complex, the enzyme should remain in the "open" conformation.

MgADP was titrated into a substrate solution containing the enzyme in addition to 5-fold concentration of sugar substrate, PGA, to ensure active site saturation by ternary complex formation. Ultimately, comparison of the non-productive ternary complex titration versus the titration with the metal-nucleotide substrate, MgADP, singularly could yield invaluable insights into the energetics of the PGK reaction.

In this experiment, the ligand associated to the substrate complex with higher affinity, K_D= 0.15 mM. The ternary complex formation completed by MgADP was demonstrated to be only a slightly exothermic binding reaction, ΔH= -0.7 kcal/mol. On the other hand, the entropic contribution exceeded beyond 50% of the initial MgADP titration, ΔS=15.1 $calK^{-1}mol^{-1}$. The free energy remained consistently favorable at approximately the same level, ΔG=-5.3 kcal/mol.

The titration of MgADP into a solution containing the enzyme, PGK, and saturating sugar substrate, PGA, including sulfate determined a ligand affinity significantly tighter than even the non-productive ternary complex excluding sulfate, K_D=0.10mM. The substrate coordination yielded only a slight exothermic response ΔH= -0.5 kcal/mol while the entropic contribution of association was extremely significant ΔS=17.0 $calK^{-1}mol^{-1}$. Overall, complex formation proceeded due to a favorable free energy of association ΔG= -5.7 kcal/mol.

ATP-Analog Ternary Complex Formation

The most unique type of ligand utilized in this study was the metal-nucleotide ligand titrated into PGK solution involved the ATP analog MgAMP-PCP. The ligand is non-hydrolysable, allowing for direct examination of the ternary complex including both the sugar substrate and metal-triphosphate nucleotide substrate, without catalytic turnover. The ligand-substrate association released a moderate amount of heat when compared to the other complex formations, ΔH=-1.1 kcal/mol. Surprisingly, however, the affinity for the MgAMP-PCP substrate remained similar to both the MgATP and MgADP additions to PGK creating binary complexes, K_D=0.23 mM. Lastly, the entropic contribution determined resembled that of the non-productive ternary complex, ΔS=13.1 $calK^{-1}mol^{-1}$. The reaction remained favorable producing a strongly occurring reaction ΔG=-5.0 kcal/mol.

The non-hydrolysable metal-nucleotide substrate bound the ternary complex in the presence of sulfate with significantly decreased affinity versus the MgAMP-PCP titration excluding sulfate, K_D= 0.7mM. However, the association of the non-hydrolysable ligand in the presence of sulfate released a very similar amount of heat when compared to the enthalpy of binding of the titration without activating sulfate, ΔH= -1.2 kcal/mol. The entropic contribution determined was reduced mildly, ΔS=10.5 $calK^{-1}mol^{-1}$ over the corresponding titration excluding sulfate. The reaction was again exergonic and the final Gibbs free energy was determined, ΔG= -4.4 kcal/mol.

Sugar Substrate Binding to PGK

Examination of the association of the triose substrate to PGK was significantly more complicated than the metal-nucleotide substrate. As previously mentioned, PGA binds to the N-terminal lobe of the enzyme in a positively charged region labeled the "basic patch". Mutational studies have been performed at length along the sugar substrate binding pocket shedding some light on the catalytic or regulatory role of residues: Arg21, Arg38, Arg65, Arg 121, Arg168, His62, His167, and His170 (Walker et al., 1991; Sherman et al., 1990; Fairbrother et al., 1989; Sherman et al., 1991; Barber et al., 1993). Examining the complex utilizing isothermal titration calorimetry should provide additional characterization through the thermodynamics of the ionic interactions of the sugar substrate binding.

The preparation of ligand solutions remained consistent with the previous metal-nucleotide experiments. The ligand was again titrated into the cell at a ratio of 50:1. However, unlike the previous metal-nucleotide titrations, the opening injection of the sugar-substrate was endothermic; furthermore, the isotherm saturated rapidly in a more endothermic direction near a molar ratio of one. Subsequent titrations of the ligand solution into the cell beyond a molar ratio of 1 provided a very minor, yet consistently decreasing signal. Saturation did not occur within the reading frame of the isotherm and likely extends five-fold beyond the limit of the experiment (see Figure 9). As a result it was impossible for the software to perform any non-linear fitting.

It was previously proposed the heat of buffer was responsible for the endothermic nature of this titration. That most of the heat consumed upon the binding of PGA arose from buffer protonation effects and not directly as a consequence of the binding interactions themselves (McAuley-Hecht and Cooper 1993). It was therefore concluded that the binding of PGA to PGK was in fact, largely exothermic. However, this phenomenon was not demonstrated. Under no experimental conditions in which the sugar substrate coordination with PGK was studied here, did the association of ligand yield an exothermic response. Furthermore, all previous thermodynamic work examining the addition of sugar substrate to PGK determined that binding was indeed endothermic (Hu and Sturtevant, 1997). While acknowledging that these binding parameters are indeed sensitive to the pH of the system as well as the presence of anions, the buffer correction for all PGA titrations was negligent, and far from exceedingly exothermic.

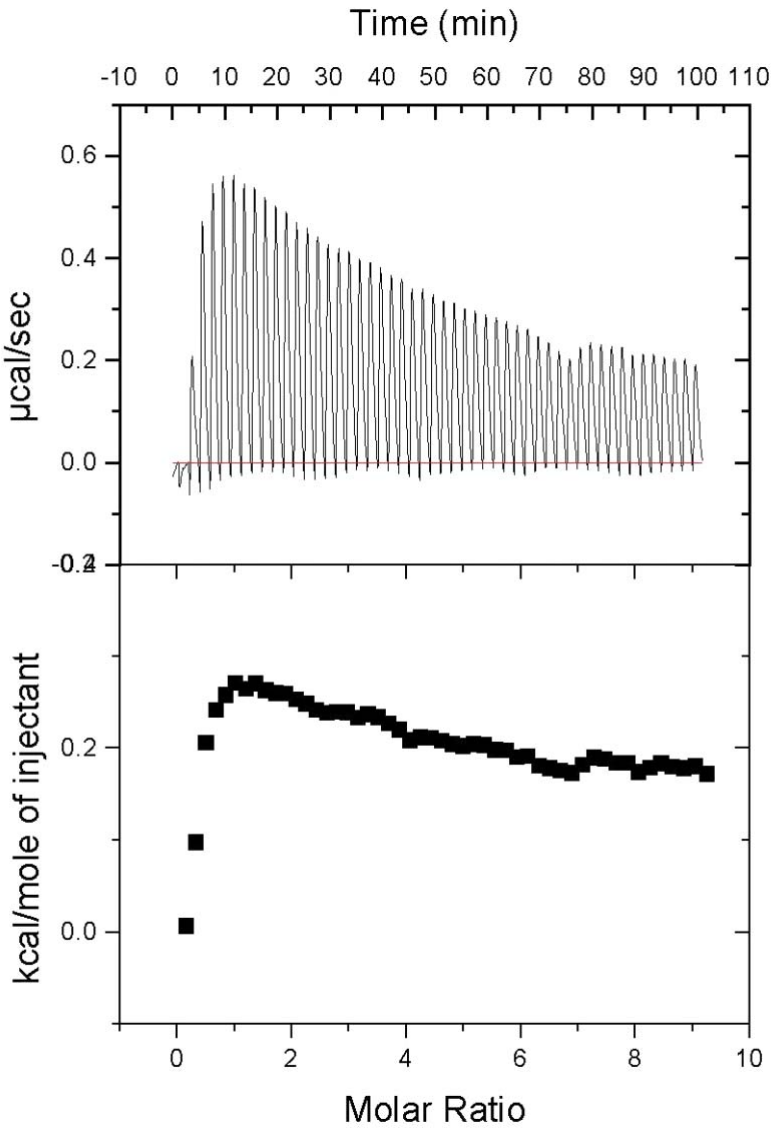

Figure 9 **Binding of 3-PGA to PGK.** A calorimetric titration of 5 µL aliquots of 25 mM 3-Phosphoglycerate into 0.5 mM Phosphoglycerate Kinase in 50 mM Tris, pH 8.0.

Chapter 4: Discussion

Isothermal Titration Calorimetry

The wealth of knowledge available from the thermodynamic characterization of a reaction by ITC is still not fully appreciated. The thermodynamic information derived from a single study includes contributions from all of the events that could occur along the journey of molecules from the free to the bound state of the observable complex. As a result, the thermodynamic contributions determined do not simply include the energetics from the creating or breaking of non-covalent bonds between interacting molecules of interest, but also the effects of the dehydration of the binding surfaces; the protonation/deprotonation of reactive groups; binding or removal of cations/anions; or dissociation/association of the reactive molecular components (Cliff and Ladbury, 2003).

The Thermodynamics of Metal-Nucleotide Substrate Binding

The technique of Isothermal Titration Calorimetry is primarily utilized to characterize the thermodynamics of simplified binding events. However, the ambiguous nature of the catalytic and regulatory binding interactions of PGK and its substrates convolute the overall thermodynamic characterization of the enzyme reaction. Previous kinetic studies indicated that the PGK enzyme associates to a single molecule of metal-nucleotide substrate and sugar substrate at a high affinity catalytic binding pocket (Raznikiewicz, 1967). However, additional coordination to a variety of anions, as well as both catalytic

substrates, has also been demonstrated (Scopes, 1978b). Both regulatory and catalytic substrates exist as multivalent anions, which have been proven to bind to the enzyme at multiple regions local and distant to the active site (Khamis & Raznikiewicz 1981). As a result, comparison of the data yielded a great deal of discrepancy within the thermodynamic parameters when considering the stoichiometry of the reaction (see tables 1 and 2). The data analysis of the metal-nucleotide titrations proved to be quite challenging. Specifically, the nature of PGK to bind to multiple ionic molecules along different regions of the enzyme clouded the overall binding characterization of the protein.

All titrations and subsequent non-linear fittings were performed with the intention of achieving the least squared differential between the theoretical and actual data points, or chi-square value. It is of paramount importance to note that each titration was performed at least twice and in several instances on three occasions. However, several of the experiments in which the stoichiometry of the reaction was permitted to vary without boundary, failed to achieve a chi-square minimum. As a result, the number of ligands bound to the enzyme varied significantly and often reached an asymptotic value which rendered the data ineffectual (see table 2). When examining ITC data, the experimental binding isotherm can be characterized by the value c, which is a product of the association constant of the reaction and the concentration of the macromolecule if the stoichiometry is 1:1. The majority of c values of the titrations in this study remained within the recommended range of 1 to 1000. However, several of the experiments

Table 1 Thermodynamic contributions of metal-nucleotide complexes titrated into PGK solutions utilizing a fixed number of binding sites.

TItration		C value	Conformation	ΔH cal/mol	K_D mM	Sites Occuppied	ΔS $calK^{-1}mol^{-1}$	ΔG kcal/mol
MgATP into PGK w/Sulf	n=2	1.32	open	-1636 ±87.0	0.38 ±0.19	0.8	8.14 ±4.90	-4.10 ±1.49
MgATP into PGK w/out Sulf	n=3	1.72	open	-2259 ±1181	0.29 ±0.17	0.8	8.95 ±2.38	-4.97 ±0.46
MgADP into PGK w/Sulf	n=2	0.81	open	-2736 ±641	0.62 ±0.01	0.9±0.1	5.60 ±1.82	-4.44 ±0.09
MgADP into PGK w/out Sulf	n=2	1.28	open	-1700 ±236	0.39 ±0.15	0.8	10.1 ±1.6	-4.76 ±0.25
MgADP into PGAPGK w/Sulf	n=2	5.00	open	-495 ±6.00	0.10 ±0.06	0.8	17.02 ±1.28	-5.65 ±0.39
MgADP into PGAPGK w/out Sulf	n=2	3.33	open	-713 ±115	0.15 ±0.10	0.8	15.12 ±0.52	-5.29 ±0.04
AMP-PCP into PGAPGK w/Sulf	n=2	0.68	open	-1201 ±318	0.73 ±0.17	0.8	10.45 ±1.53	-4.35 ±0.14
AMP-PCP into PGAPGK w/out Sulf	n=2	2.17	open	-1053 ±113	0.23 ±0.03	0.8	13.12 ±0.58	-5.03 ±0.06

Table 2 Thermodynamic contributions of metal-nucleotide complexes titrated into PGK solutions utilizing a variable number of binding sites.

Titration		C Value	Conformation	ΔH cal/mol	K_D mM	Sites Occuppied	ΔS $calK^{-1}mol^{-1}$	ΔG kcal/mol
MgATP into PGK w/Sulf	n=2	1.25	open	-2831 ±658	0.40 ±0.18	0.79±0.20	6.36±3.12	-4.76 ±0.29
MgATP into PGK w/out Sulf	n=1	1.08	open	-3681	0.465	0.375	3.03	-4.599
MgADP into PGK w/Sulf	n=1	0.67	open	-12161	0.746	1.89	10.1	-15.221
MgADP into PGK w/out Sulf	n=2	0.60	open	-1345 ±106	0.84 ±0.60	1.27±0.47	10.27 ±1.54	-4.48 ±0.55
MgADP into PGAPGK w/Sulf	n=2	3.85	open	-3144 ±1375	0.13± 0.04	0.175±0.07	7.53 ±3.88	-5.42 ±0.20
MgADP into PGAPGK w/out Sulf	n=2	2.78	open	-1923 ±1531	0.18 ±0.09	0.66±0.46	11.08 ±6.23	-5.28 ±0.36
AMP-PCP into PGAPGK w/Sulf	n=2	0.72	open	-1679 ±1014	0.69 ±0.04	1.03±0.70	8.94±3.27	-4.38 ±0.02
AMP-PCP into PGAPGK w/out Sulf	n=3	1.92	open	-3025 ±1618	0.26 ±0.07	0.50±0.2	6.70±5.30	-4.97 ±0.15

revealed a c value <1 signaling an isotherm which has a very broad transition and is difficult to analyze (Pierce et al., 1999). This data is perhaps indicative of a system in which many binding events are occurring simultaneously. One of the limitations of the ITC experiment lies with the ability to differentiate between multiple enthalpies. The heat change detected between subsequent titrations is a global entity encompassing all associations and interactions within the buffering system as well as the substrate-ligand interactions. Considering the extreme reliance on ionic interactions for binding and the polyionic nature of the macromolecule, a global enthalpy will challenge the ability of the ITC technique to isolate the desired binding event.

MgATP Titration into PGK Solution

Extensive structural work has been performed detailing the domain translational movements of PGK upon titration with MgATP; existing domain interactions are strengthened and weakened, created and removed yielding a slightly compacted conformation towards the catalytically active form. A natural result of this association to the metal-nucleotide is an alteration of the active site environment and likely a reorganization of solvent (Roustan et al., 1979; Fairbrother et al., 1990).

The coordination of each of the phosphates has been demonstrated to be a critical interaction for the instigation of the hinge-bending mechanism (Pappu et al., 1994; McPhillips et al., 1996). Coordination of the β and γ phosphates but not α, is suggested to be necessary for the initiation of domain closure and therefore the presence of the γ

phosphate is critical for the interactions necessary for catalysis. Mechanistically, interaction of the γ phosphate with the metal ion simultaneously with the sugar substrate has been hypothesized to be a possible trigger mechanism for the hinge-bending motion (Pappu et al., 1994). It is believed this mechanism involves the translation of helix XIV into a locked position across the face of the separate domains creating a hydrogen bonding network ultimately inducing domain closure of the enzyme (Bernstein et al., 1997).

Previous thermodynamic studies suggested that the binding of MgATP to PGK was a reaction driven largely by the sizeable enthalpic contribution (Hu and Sturtevant, 1987). However, these results were determined by the indirect method of Differential Scanning Calorimetry and calculated van't Hoff enthalpies. While a consistently exothermic binding reaction was observed in this study, the complete thermodynamic characterization determined that an entropic contribution must also be considered and likely plays a larger role in complex formation than first anticipated. This observation is consistent with the previous structural data as a slight compaction of the enzyme and alteration of the active site environment could be indicative of a solvent expulsion in addition to the aforementioned reorganization. While the residues most closely implicated in the binding of the metal-nucleotide substrate are ionic (D372 and E398) and the affinity of the metal-nucleotide is directly related to the magnitude of charge of the substrate (Wrobel and Stinson, 1978), evidence of the adenine ring of the nucleotide substrate binding to a hydrophobic region along the carboxy-terminal domain of the enzyme was determined (Watson et al., 1982). Therefore, hydrophobic interactions may

contribute to the stabilization of the enzyme-metal-nucleotide complex. Ultimately, in addition to the release of heat, the driving force of MgATP association to PGK may include a much larger entropic significance in the form of solvent expulsion from the catalytic core of the enzyme by the incoming metal-nucleotide substrate than first anticipated.

MgADP Titration into PGK Solution

MgADP lacks the γ phosphate of MgATP. It was suggested by Pappu et al., (1994) that metal coordination only to the β– and γ– phosphates was required for domain closure and catalysis. In this orientation with the coordinated metal ion, the phosphate is able to extend across the interdomain cleft close enough to the sugar molecule for phosphoryl transfer. This may then explain the lower affinity of MgADP to the enzyme compared to MgATP and the lower enthalpy of binding to the enzyme due to the lack of interactions of the γ– phosphate.

Any number of failed interactions could account for subtle thermodynamic differences between MgATP and MgADP titrations. In addition, a variation in entropic contributions arises with the addition and removal of sulfate from the complex. The lack of catalytically-introduced hydrogen bonding network among other induced interactions of the hinge-bending motion is likely responsible for the variability of entropy.

Non-productive Ternary Complex Formation: MgADP Titration into PGA-PGK Solution

Specific interactions are necessary in order to initiate the hinge-bending motion necessary for catalysis (Pappu et al., 1994; McPhillips et al., 1996; Bernstein et al., 1997; Pappu et al., 1997). According to the recent crystal structure solved by Bernstein et al., (1997) it was postulated that the correct orientation of the bound sugar substrate "primes" the active site of the enzyme in order to initiate the interactions necessary for domain closure by perhaps the helix translation/hydrogen bonding mechanism mentioned previously. While the ternary complex investigated here does not contain the acyl phosphate necessary for transfer, many of the same ionic interactions and hydrogen bonding networks should alter the thermodynamics from the binary complex, MgADP titrations.

A significant increase in entropy was determined upon formation PGK-MgADP-PGA when compared to PGK-MgADP complex. The increased entropy is likely to be the result of a more occluded active site and subsequent compaction of the enzyme resulting in the expulsion of solvent necessary for binding. These results are consistent with neutron scattering studies which detailed a decrease in the radius of gyration upon non-productive ternary complex formation (Henderson et al., 1994). However, the neutron scattering studies were performed with a catalytically relevant substrate that yields a single turn reaction. MgADP on the other hand, cannot induce the same structural changes and thus the substrates still remained too distant for direct phosphate transfer, thus the enzyme maintained the catalytically inactive, "open" conformation.

Accompanying the increase in entropy of formation, there was a sizeable decrease in the enthalpic contribution as well as increase in binding affinity of almost an order of magnitude versus MgATP binding. The inverse changes in entropy and enthalpy perhaps indicate that ternary complex formation is an event which relies much more heavily on entropy as its driving force than the binary complex formations. In particular, the translation of helices suggested by Bernstein et al., (1997) could be a single example of the multiple intramolecular movements inside the catalytic core of the enzyme as well as along the periphery. While the aforementioned translation is domain oriented, the enlarged entropy of the reaction is perhaps indicative of increased side chain mobility at the binding site (Jelesarov and Bosshard, 1999). The increased movement of both the amino acid side chains as well as the separate domains of the protein likely result in the increased entropy of the binding reaction of the non-productive ternary complex.

The intramolecular interactions created by the bound sugar substrate are also likely responsible for the increased binding affinity of the metal-nucleotide substrate. A direct ionic interaction between the amino terminal domain regions involved in sugar substrate binding and the associating metal-nucleotide are unlikely to significantly increase the binding affinity due to the extensive distance maintained between the two domains. However, it is possible that as part of the slight domain closure upon sugar substrate binding, unforeseen residues are exposed at the metal-nucleotide binding pocket increasing hydrophobic interactions proximal to the adenine ring as well as perhaps hydrogen bonding and ionic interactions along other portions of the metal-nucleotide

substrate. Clearly, the addition of bound sugar substrate greatly altered the thermodynamics of non-productive ternary complex formation.

Ternary Complex Formation with a Non-Hydrolysable Metal-Nucleotide Analog: The Binding of MgAMP-PCP to PGA-PGK Solution

The titrations of MgADP into PGK with 5-fold PGA provided valuable insights into the secondary effects of ternary complex formation. However, non-productive ternary complex formation is limited as it does not contain a hydrolysable phosphate linkage within the metal-nucleotide or the sugar substrate. On the other hand, the previous titration did provide some preliminary clarity of the "priming" process accompanied by sugar substrate binding. Furthering the data from the non-productive ternary complex titrations, the utilization of the ATP analog AMP-PCP supplemented the overall thermodynamic characterization by fulfilling the ternary complex with the non-hydrolysable phosphate bond previously lacking. However as a result of the P-C-P linkage of the terminal phosphate, the substrate is non-hydrolysable, and no catalysis will occur. Still, the metal-coordinated ATP analog substrate more closely represented the native substrate than any other previous studied polynucleotide molecule and the γ phosphate will likely be able to imitate many of the intermolecular interactions necessary to initiate the hinge-bending motion. While novel thermodynamic data should become available from this titration, the enzyme will still remain in the "open" conformation in the crystal structure (McPhillips et al., 1996).

Examination of the thermodynamic contributions of the ternary complexes produced somewhat surprising results. While it would seem that the full thermodynamic profile of the ternary complex formation with MgAMP-PCP should be more representative of the non-productive ternary complex formation over either of the binary complex titrations, in reality, the thermodynamic contributions lie somewhere in between. This data is again indicative of a varying magnitude of enthalpy and entropy driving the reaction. The inconsistencies of the thermodynamic contributions are a result the intramolecular interactions introduced into the catalytic core of the enzyme upon ternary complex formation. The increase in enthalpy versus the non-productive ternary complex is consistent with ionic interactions playing an expanded role in binding. While on the other hand, the still sizeable entropic contribution also signals the enhanced hydrophobic interactions revealed through the interactions and movements initiated by ternary complex formation.

Comparison of Enthalpic Contributions of Binary and Ternary Complex Formation

The enthalpy change measured by ITC is a representative value encompassing the whole experimental system. It is the total heat released or absorbed within the calorimetric cell upon each addition of the ligand into the substrate solution. The total heat change detected by the instrument includes contributions from: the heat of dilution of the ligand into buffer, heat resulting from the titration of ligand solution into substrate solution at

differing temperatures, or effects of mixing two solutions of slightly different buffer compositions (Jelesarov and Bosshard, 1999). Therefore, it is imperative to exhaust all necessary means in order to simplify the heat effects of the binding reaction.

Very little thermodynamic work has been performed comparing the differences in the magnitude of heat released upon the formation the binary and ternary PGK complexes. Analysis of the ITC data is reliant upon the discovery of enthalpic trends within each series of experiments. There are several distinct consistencies which arise from the examination of data when the stoichiometry of the reaction was maintained at the previously established ratio of 1:1. In agreement with published data, the binding of all metal-nucleotides to PGK is an exothermic reaction (Hu and Sturtevant, 1986), releasing 0.5 kcal/mol of heat per mole to almost 2.7 kcal/mol, in these titrations. The association of all binary complexes elicited quite a bit more heat than the ternary complexes containing PGA as part of the enzyme solution. Exact interpretations of enthalpic data have often times eluded the investigator due to the multiple sources of heat release during complex formations such as PGK. However, it has been agreed upon that solvent reorganization accounts for a very large portion of the change in heat released, ΔH (Connelly et al., 1993; Chervenak and Toone, 1994) and indeed, extended hydrogen-bond networks at the coordination interface in the absence of water molecules can make the enthalpy change more favorable (Bhate et al., 1994). In this study, a significantly greater enthalpy change was calculated upon binary complex formation versus ternary complex formation. It appears illogical to conclude that binary complex formation will have a greater effect on the reorganization of solvent along the binding interface of the

interdomain cleft of the enzyme. However, if one takes into account that sugar substrate was introduced to the enzyme solution prior to the calorimetric titrations, the decrease in enthalpy change can be explained. It is likely the addition of the sugar substrate altered a generous portion of the solvent within the catalytic core of the enzyme preceding the titration of interest. Presumably, the macromolecule solution containing buffer and enzyme alone has no solvent occlusions along the interdomain cleft of the active site. Therefore, the reorganization of solvent reflected by the diminutive change in heat of the metal-nucleotide titrations into PGK solution containing PGA was considerably lessened by the previously bound sugar substrate.

As previously mentioned, the thermodynamic value of enthalpy is a global entity which encompasses the heat expelled or consumed along the entire interface of the macromolecule. The difference in heat released between biphosphate and triphosphate complex formations could be a direct result of the presence of the γ phosphate. The additional phosphate group may introduce new ionic or hydrogen bonds altering previously established interactions. Crystal data of pig and yeast PGK suggested that hydrogen bonding linked to the translation of helix XIV upon metal-nucleotide binding plays a key role in domain closure (Mas et al., 1996; McPhillips et al., 1996). Furthermore, a small difference in domain closure was determined by neutron scattering studies when binary and ternary complex formations were compared clearly indicating dissimilarities in their respective active site environments after binding (Henderson et al., 1994). While full domain closure of the enzyme was not witnessed by crystallographic analysis without an amino acid insertion, partial compression of the molecule was

determined even upon non-productive ternary complex formation. The differences in enthalpy between the binary and ternary complexes could be attributed to the "pulling" of the PGK molecule by the bound substrates resulting from the strengthening and weakening of existing bonds. With a molecule such as PGK, isolating the enthalpic contribution from the individual substrates and their thermodynamic consequences proved very difficult to determine.

Comparison of Entropic Contributions of Binary and Ternary Complex Formation

Entropy of binding can be calculated from the determined values of Gibbs' free energy, heat capacity, and enthalpy. The change in entropy of a complex relative to the unbound molecules is largely a result of the hydration effects because the entropy of hydration of polar and non-polar groups is significant and there is a sizeable reduction of water accessible surface upon binding (Jelesarov and Bosshard, 1999). Therefore, when complex formation occurs, the overall entropy change is often large and positive. Each binary titration possessed a significantly lesser entropic contribution than its ternary counterpart. Previous small angle X-ray and neutron scattering studies of the binary and ternary complexes have suggested that a conformational change occurs upon the binding of both the sugar substrate and metal-nucleotide substrate (Pickover et al., 1979; Henderson et al., 1994) and the radius of gyration of PGK is reduced more upon formation of ternary complexes when compared to binary complexes. The enzyme is oriented in a more compact conformation upon ternary complex formation, which is

consistent with the proposed hinge-bending motion necessary for catalysis and likely to result in a greater amount of solvent displacement from the interdomain cleft of the enzyme. The thermodynamic data from this study, at first glance, looks counter intuitive because one would expect that individual molecules would possess more freedom in the binary complex, hence more positive entropy. However, experimental results show that the gain of entropy is higher in the ternary complexes. This can be explained if one assumes that the formation of the ternary complex expels more water molecules from the binding interface in the ternary complex then the binary complex. Then the entropy gained by the water molecules would explain the larger entropy observed from the ternary complex formation. This conclusion is in agreement with Jelesarov and Bosshard (1999) who suggested that a positive ΔS is a strong indication that water molecule have been expelled from the complex interface.

In addition to the expulsion of water from the active site as a result of domain closure, hydrophobic interactions may also perform a key role in the initial binding of the metal-nucleotide substrate. A recent crystal structure of pig muscle PGK determined the adenine ring of the metal-nucleotide substrate was bound in a small hydrophobic slot along the surface of the carboxy-terminal domain of the enzyme which is consistent with initial PGK crystal structures (Watson et al., 1982; May et al., 1996). The thermodynamic contribution of the binding of the adenine ring separately from the entire substrate would be extremely difficult to determine as the molecule relies on ionic interactions primarily for association. However, it is likely that organization of solvent

away from the adenine ring binding pocket does influence the overall entropy of binding of the substrate.

Another factor to consider when drawing conclusions surrounding the entropic contribution of complex formation is the degree of mobility of the amino acid sidechains directly involved in coordination upon binding. An unfavorable contribution to the energy change likely originates from the reduction of side chain movement and overall flexibility of the interacting molecules (Jelesarov and Bosshard, 1999). Evidence of multiple coordination sites perhaps simultaneously, for the metal-nucleotide substrate along the carboxy-terminal domain would suggest a larger degree of freedom of the interacting side chains and an increased entropic contribution accompanied by a decrease in the enthalpy of binding.

The full significance of the increased entropy of ternary complex formation over binary complex formation cannot be understood without examining the entire thermodynamic profile. Considering the relative consistency of the free energy regardless of the complex titration, it is imperative to recognize the enthalpy-entropy compensation and its significance. It was previously suggested by Hu and Sturtevant (1993) that binding of the metal-nucleotide alone was largely driven by the favorable enthalpy change. However, ternary complex formation was characterized by an extremely favorable entropy change. Therefore, it is likely that hydrophobic interactions and solvent exclusion from the active site play a much larger role in ternary complex formation versus binary complex formation.

The Effects of Activating Sulfate on the Thermodynamics of PGK Complex Formation

The influences of sulfate on PGK complex formation range from kinetic activation to structural changes. In almost every series of titrations, the introduction of sulfate into the PGK solution decreased the affinity of the ligand for the enzyme complex with the exception of MgADP. Several hypotheses exist for the decreased interaction of the substrate and enzyme in the presence of sulfate. Previous kinetic studies determined that the catalytic mechanism is strongly linked to the binding affinity of the substrates. Scopes postulated that the release of substrate from the catalytic core likely represents the rate-limiting step of the PGK reaction (Scopes, 1978b) and thus affinity for the enzyme is of vital importance. Ionic interactions play a key role in complex formation as evidenced by the relationship between binding affinity and overall charge of the substrate (Wrobel and Stinson, 1978). This study determined that as the charge of the substrate increased, the binding affinity increased significantly as well. In addition, an induced hydrogen bonding network is proposed to play a major role in the initiation of domain closure (Bernstein et al., 1997). Finally, most of the coordinating residues implicated by crystallographic analysis are polar and likely interact via ionic bonding (Watson et al., 1982; Pappu et al., 1994; McPhillips et al., 1996; and Pappu et al., 1997). The decreased affinity for the enzyme by substrate with the introduction of sulfate represents a possible shielding effect lessening the ionic interaction and subsequent coordination of the substrate to the enzyme. If indeed the binding and release of substrate is the critical step

for PGK activity, then disruption of the enzyme-substrate interactions is likely a source of an increased Michaelis rate constant.

Comparison of Thermodynamic Contributions Resulting From Fixed Number of Binding Sites versus Variable Number of Sites

Comparison of the data presented when examining the number of sites bound to the substrate does not evoke similar consistencies. The variability of binding of the PGK enzyme is evidenced by the stoichiometry calculated which ranged from 1.89 sites saturated to 0.175. Furthermore, as previously mentioned the number of repetitions of each titration was decreased from the previous data set due to the fact that in several instances, the stoichiometry failed to approach a single substrate-ligand association and yielded thermodynamic parameters which were not possible. The ambiguous binding nature of both the regulatory and catalytic pockets allows for a number of different substrates to coordinate to either type of site. As a result, it is conceivable that a catalytic substrate could bind to both the regulatory site and active site. Therefore, the seemingly inconsistent data did not simply represent substrate association to a single binding pocket; instead, it more likely indicated the combination of several binding events both catalytic and regulatory coordinated to different regions of the PGK molecule. Also, it is likely that the thermodynamic contributions represented a binding and transfer of substrate from one site to another. This is consistent with solution studies by Serpersu et al., (2002) and Fairbrother et al., (1990b) indicating the catalytic and regulatory binding sites are in close proximity to each other and perhaps even overlap.

Additionally, considering the "hinge-bending" nature of the protein and the slight domain closure observed upon binary complex formation (Henderson et al., 1994), it is likely that binding of regulatory and catalytic substrates will alter the orientation of the separate domains of the enzyme leading to the introduction of an induced-fit binding region and assist in the compaction of the enzyme from the "open conformation" to the catalytically active "closed conformation." Ultimately, allowing the stoichiometry of the thermodynamic data to progress without limitation does not sufficiently eliminate enough ambiguity of binding of the substrates to the PGK molecule in order to focus on the catalytic pocket singularly.

Sugar Substrate Binding to PGA

The thermodynamics of the binding of the sugar substrate to the PGK enzyme has remained elusive. Therefore, while it has been widely conceded that metal-nucleotide binding is exothermic and largely enthalpically driven, differing theories exist regarding the thermodynamics of sugar substrate binding. The findings from this study are in contrast to results by McAuley-Hecht and Cooper (1993). Indirect thermodynamic studies of the PGK complex association utilizing Differential Scanning Calorimetry exhibited an endothermic characterization of sugar substrate binding (see Table 3). On the other hand, previous direct calorimetry studies have suggested that association of

Table 3 Thermodynamic contributions of PGK and its substrate complexes determined by Differential Scanning Calorimetry (Hu, C. Q. & Sturtevant, J. (1987) *Biochemistry 26*, 178-182).

Table I: Enthalpy of Binding of MgATP, MgADP, and 3-PG to PGK

temp (°C)	ΔH_b (kcal mol^{-1})		
	MgADP	MgATP	3-PG
9.95	−7.86 ± 0.19[a] (8)[b]	−7.70 ± 0.04[a] (8)[b]	−0.77 ± 0.01[a] (8)[b]
17.50	−8.59 ± 0.62 (9)	−6.80 ± 0.21 (7)	2.01 ± 0.08 (7)
25.00	−9.36 ± 0.77 (9)	−7.56 ± 0.01 (8)	3.74 ± 0.08 (8)
32.00	−12.03 ± 0.33 (8)	−7.43 ± 0.33 (8)	2.07 ± 0.02 (8)

[a] Uncertainties are expressed as the standard error of the mean.
[b] The numbers in parentheses indicate the number of measurements.

PGA to PGK evoked a sizeable exothermic reaction (McAuley-Hecht and Cooper, 1993). In reality, the overall global enthalpy determined by McAuley-Hecht and Cooper (1993) was significantly endothermic; however, the heat change was attributed to the buffer dilution and protonation effects, which were not demonstrated. The studies performed here included titrations at a variety of sugar substrate concentrations which elicited consistently endothermic responses, in agreement with the results of Hu and Sturtevant (1987).

Non-linear fitting of sugar substrate addition to PGK was indeterminable due to the nature of the isotherm. Specifically, ITC relies on the principle that a change in the heat as the result of a binding reaction occurs in the solution environment until all binding sites of the macromolecule are saturated and no heat difference between subsequent titrations remains. Even well beyond the extended readable frame of the experiment, this saturation was never achieved, rendering the fitting software ineffectual. This phenomenon could be the result of several factors. As previously mentioned the sugar substrate possesses very similar anionic characteristics as sulfate and other molecules capable of activating the PGK reaction and can bind proximal to the catalytic core or at regulatory binding regions remote from the active site creating a very ambiguous binding model (Raznikiewicz and Jannson, 1973). Furthermore, it was demonstrated that a strong relationship exists between the charge of the substrate and the affinity of the molecule for PGK (Wrobel and Stinson, 1978). Therefore, the polyionic nature of the enzyme to associate multiple substrates in both a catalytic and regulatory function allows for

regulation by numerous types of molecules while also creating a very convoluted binding characterization.

Upon sugar substrate binding, the PGK molecule has also been demonstrated to undergo a conformational shift which will introduce numerous different thermodynamic alterations in the PGK complex (Pickover et al., 1979; Henderson et al., 1994). In addition, the orientation of the substrate within the binding pocket could vary greatly depending upon the presence of excess substrate or regulatory molecules contained within the Active site environment. Therefore is it likely the separate domains of the enzyme as well as the specific side chains along the binding regions themselves experience a conformation shift altering the tertiary structure of the enzyme upon coordination to substrate. Finally, as discussed earlier, there may be as many as six binding sites (and perhaps more) in which both the sugar substrate and the regulatory substrate molecules can associate along the PGK enzyme (Scopes, 1978). The combination of the polyionic nature of the enzyme and the ionic similarities of the different types of substrates are the driving force behind the convoluted binding pattern of the enzyme.

Conclusions

The studies presented here have shed some light into the thermodynamics of formation of the various PGK complexes. The association of all metal-nucleotide substrates investigated was exothermic, generating heat ranging from 0.5 kcal/mol to 2.8 kcal/mol.

Binary complex formation was determined to release a greater magnitude of heat than ternary complex formation. Considering the polyionic character of the enzyme to associate many different types of molecules and the polar nature of the metal-nucleotide substrates, ionic interactions play a key role in binary complex formation which is largely characteristic of an enthalpically driven reaction. The sizeable heat generated upon binding is likely the result of a strengthening and weakening of non-covalent interactions as well as solvent reorganization along the enzyme-metal-nucleotide complex interface.

Ternary complex formation on the other hand, was determined to rely much more heavily on the entropy of formation in order to drive the reaction. While the reaction remained exothermic, the magnitude of heat released was significantly diminished. In order to compensate for the relatively minor difference in overall free energy of formation, the entropy of association increased dramatically to as much as 17.02 $calK^{-1}M^{-1}$. The overwhelming entropic compensation of the reaction is suggestive of a complex which is at least moderately reliant on hydrophobic interactions. In addition, solvent exclusion from the active sight is a likely consequence of binding. Indeed, according to crystal structures, the adenine ring of the nucleotide lies within a hydrophobic slot along the carboxy-terminus of the enzyme (Watson et al., 1982). The further occluded catalytic core of the protein by the existing sugar substrate must displace a greater amount of solvent from the active site than the metal-nucleotide alone. As a result, ternary complex formation is significantly more dependent upon spatial accessibility than simply the ionic interactions of binary complex formation.

The affinity of the metal-nucleotide substrates proved to be difficult to determine. According to the preliminary solution studies and extensive crystallographic analyses, the stoichiometry of the reaction remains simple; one molecule of each substrate combined with the enzyme yields a single molecule of each product. However, the polyionic nature of the protein allows for an extremely complex binding characterization. The metal-nucleotide substrates demonstrated greatest affinity for the enzyme during ternary complex formation. This is likely due to the induced interactions from the bound sugar substrate which has been proposed to include a small domain closure as well as a hydrogen bonding network which aids in "priming" the active site for the "hinge-bending" motion (Bernstein et al., 1997).

In addition, the enzyme was proven to bind various different types of ionic molecules including excess substrate (Wrobel and Stinson, 1978). Therefore, when determining the thermodynamic parameters, it was necessary to maintain the number of number of bound sites of the enzyme. Without this binding consistency, the separate thermodynamic contributions were often indeterminable. These thermodynamic fluctuations simply highlighted the ability of the enzyme to associate ionic molecules to as many as six binding regions within the catalytic core and along the out surfaces of the globular domains.

Finally, sulfate has long been known to greatly enhance the activity of the PGK reaction. The thermodynamics of the reaction do alter upon introduction of sulfate and perhaps elucidate several possible mechanisms of activation. In almost every titration, the

addition of sulfate decreased the binding affinity of the substrate for the enzyme. It was postulated that the release of the substrate was the rate-limiting step of the reaction (Scopes, 1978). Therefore, considering that substrate binding to the enzyme relies heavily on ionic interactions, it is highly possible the presence of sulfate creates a "shielding" effect which facilitates the release of the substrate and consequently activates the reaction. In addition, an increase in the entropic contribution of formation was determined upon the inclusion of sulfate into the titration. Sulfate has been demonstrated to cause a slight compaction of the enzyme consistent with singular substrate binding (Henderson et al., 1994). Furthermore, there are at least two binding regions which can associate sulfate within the catalytic core of the protein (Fairbrother et al., 1990; Serpersu et al., 2002). As a result, the binding of sulfate at or adjacent to the active site will clearly have an effect on the immediate environment and likely trigger the expulsion of solvent, which is consistent with an increase in entropy.

The thermodynamic parameters of sugar substrate binding proved to be more elusive. While it can be conceded that binding is an endothermic event, the exact thermodynamic values were unable to be identified. The culprit remains the polyionic nature of the protein to bind various types of molecules at different sites within the interdomain cleft and along the periphery of the molecule. Inherently, the enthalpy of formation is a global entity of all the binding events simultaneously.

Overall, the utilization of the technique of Isothermal Titration Calorimetry has shed some light into the thermodynamics of the "hinge-bending" catalytic mechanism

surrounding the enzyme phosphoglycerate kinase. This work illustrated the value as well as the inherent complexity of the direct thermodynamic measurement of ITC. Specifically, the polyionic nature of the various substrates as well as the binding regions created a very convoluted thermodynamic profile.

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Vita

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